PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re the Application of:	)	Group Art Unit:
	)	
NETT et al.	)	Examiner:
	)	
Serial No.:	)	FIRST PRELIMINARY AMENDMENT
	)	
Filed: Herewith	)	"EXPRESS MAIL" MAILING LABEL NUMBER: EL 767781926 US DATE OF DEPOSIT: January 21, 2002
	)	
Atty. File No.: 2730-97-CON	)	I HEREBY CERTIFY THAT THIS PAPER OR FEE IS BEING DEPOSITED WITH THE UNITED STATES POSTAL SERVICE "EXPRESS MAIL POST OFFICE TO ADDRESSEE" SERVICE UNDER 37 C.F.R. 1.10 ON THE DATE INDICATED ABOVE AND IS ADDRESSED TO THE ASSISTANT COMMISSIONER FOR PATENTS, WASHINGTON, D.C. 20231.
	)	
For: "A METHOD FOR INACTIVATING GONADOTROPHS"	)	TYPED OR PRINTED NAME: Brenda Carpenter SIGNATURE: Brenda Carpenter

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

Prior to the initial review of the above-identified patent application by the Examiner, please enter the following Preliminary Amendment to amend the specification and claims as follows:

IN THE SPECIFICATION:

Please change the title of the application by deleting "GnRH ANALOGS FOR DESTROYING GONADOTROPHS" and insert therefor -- A METHOD FOR INACTIVATING GONADOTROPHS -- .

On page 1, lines 3-6, please delete "This patent application is a continuation-in-part of U.S. Patent Application 314,653 filed February 23, 1989 and also entitled "GnRH ANALOGS FOR DESTROYING GONADOTROPHS.", and insert therefor --This patent application is a continuation application of U.S. Patent Application Serial No. 09/551,933, now issued U.S. Patent No. 6,326,467;

which is a continuation of U.S. Patent Application Serial No. 09/354,295; which is a continuation of U.S. Patent Application No. 09/015,729, now issued U.S. Patent No. 6,103,881; which is a continuation of U.S. Patent Application Serial No. 08,481,128, now issued U.S. Patent No. 5,786,457; which is a continuation of U.S. Patent Application Serial No. 08/094,625, now issued U.S. Patent No. 5,488,036; which is a continuation of U.S. Patent Application Serial No. 08/094,250, now issued U.S. Patent No. 5,492,893; which is a continuation of U.S. Patent Application Serial No. 08/591,917, now issued U.S. Patent No. 5,707,964; which is a continuation of U.S. Patent Application Serial No. 08/088,434, now issued U.S. Patent No. 5,631,229; which is a continuation of U.S. Patent Application Serial No. 07/837,639, now issued U.S. Patent No. 5,378,688; which is a continuation of U.S. Patent Application 314,653, filed February 23, 1989."--

On page 12, line 2 at the beginning of the paragraph, please insert the following: --The present invention provides unique methods and compounds for regulating cells having particular hormone receptors thereon. One aspect of the present invention, described in more detail below, relates to the use of conjugates between a hormone and an agent capable of killing a cell. For example, one embodiment is directed to the use of an analog of gonadotrophin-releasing hormone (GnRHa) and compounds capable of regulating cells expressing GnRH receptors. In particular, GnRH conjugates of the present invention can be used to destroy cells expressing GnRH receptors or, alternatively, inhibit cellular function of such cells so as to regulate the continued survival of such cells and/or to regulate the secretion of particular compounds and functions of such cells. The compounds to which GnRH can be conjugated include various toxins, described in more detail below, as well as proteins capable of cleaving particular nucleic acid molecules (e.g., nucleases). In particular, the present invention includes the use of RNAse, which is capable of destroying

ribonucleic acid, conjugated to GnRH. Also included within the scope of the present invention is the use of DNAse conjugated to GnRH. As described in more detail below, various linking agents can be used to conjugate GnRH molecules to desired compounds. In addition to the nucleases that can be conjugated to GnRH, the present invention includes the use of one or more of the various toxin groups conjugated to GnRH as described hereinafter, alone or in combination with GnRH/nuclease molecules.--

On page 29, line 16, please insert the following -- Yet a further aspect of the present invention is directed to the use of bioengineered proteins conjugated with GnRH for use in regulating hormone related diseases to treat cancer, to achieve temporary and/or permanent sterilization of animals, and/or to inactivate gonadotrophs. Bioengineered or recombinant proteins, and specifically proteins having toxic moieties, offer the advantage of improved homogeneity, as compared to toxins that may be derived from other natural sources. Indeed, by using bioengineered and/or recombinant proteins having desirable cell-toxic attributes, it is believed that reduced costs of manufacture can be achieved due to the elimination of any extraction and purification procedures that would otherwise be required for recovering proteins from natural sources. In one particular aspect of the present invention, the recombinant pokeweed antiviral protein is produced and utilized which differs slightly from natural pokeweed proteins. The present inventors believe that the recombinant proteins produced by Rajamohan et al., specifically a recombinant pokeweed antiviral protein, has particular use in the present invention. Such recombinant pokeweed antiviral protein has a molecular weight of 33 kDa whereas the natural protein has a molecular weight of approximately 29 kDa. Thus, one aspect of the present invention involves the use of a hormone toxin conjugate comprised of a peptide hormone capable of binding to a GnRH receptor and at least one recombinant protein capable of

inhibition of protein biosynthesis. Such recombinant proteins include, but are not limited to recombinant pokeweed antiviral protein.

Yet a further embodiment of the present invention relates to the use of a particular linking agent, namely N-[-maleinidobutyrloxy]sulfosuccinimide ester (Sulfo-GMBS).

In another embodiment, an SH group is introduced into dLys6-GnRH through the use of 2-IT, the advantage being increased solubility of the modified peptide. --

Please insert on page 47, line 25, the following:

-- Conjugation of dK^6-GnRH with PAP.

Fourteen moles of dK^6-GnRH are dissolved in 1 ml of methanol (meOH) and then mixed (on ice) with 9.6 1 N,N-diisopropylethylamine (DIPEA). The reaction is started by addition of 17 moles of 2-iminothiolane (2-IT) dissolved in 0.5 ml of MeOH. After 2 h incubation at room temperature the solution is acidified with 7 1 CH_3COOH (100%) and dried with a stream of nitrogen. The progress of the reaction is monitored by HPLC and the product is also analyzed by MS. This reaction is faster in DMF and better yields can be achieved. MeOH is also a more convenient solvent.

Modification of PAP with N-[-maleimidobutyryloxy]sulfosuccinimide ester (Sulfo-GMBS). A 4.8 mole sample of PAP (144 mg) is dissolved in 4 ml of 0.05 M sodium phosphate, 0.1 M NaCl, 1mMEDTA, pH 7.4. The protein solution is mixed with 14.7 moles of Sulfo-GMBS dissolved in 4 ml of the same buffer. The reaction is allowed to proceed for 40-60 minutes at room temperature.

Reaction of SH-GnRH with modified PAP. Freshly prepared SH-GnRH is dissolved in deoxygenated 0.05 M sodium phosphate, 0.1 M NaCl, 1mM EDTA, pH 7.4 and mixed with freshly

prepared deoxygenated maleimibobutyryl-PAP solution. The final pH is 7.0-7.2. After incubation for 30-40 minutes at room temperature the reaction mixture is acidified to pH 4.5-5.0 with 1 M CH_3COOH, spun and the supernatant applied to a BioGel P-60 or Superdex-75 column equilibrated in 0.1 M NaCl. The fraction containing GnRH-PAP conjugate is concentrated, desalted on Sephadex G-25 (in the presence of 0.05 M NH_4HCO_3) and then lyophilized yielding 90 mg of protein. The pH 7.0-7.4 of the reaction of PAP with Sulfo-GMBS is chosen to increase reactivity of the -amino group with respect to -amino groups. Under the same conditions a higher, 70-80%, conjugation yield is obtained with ribonuclease A.

Another aspect of the present invention involves the use of "Hormone/nuclease conjugates" formed between particular hormones and particular nucleases capable of degrading nucleic acids such as RNA and DNA. Table VI, below, lists the various hormones that can be used in the present invention. The respective endocrine gland where such hormone is produced is also indicated, as well as the major function of the designated hormones.

TABLE VI

Endocrine Gland	Hormone	Major Function of:
Hypothalamus	Hypophysiotropic hormones·	Secretion of hormones by the anterior pituitary
	Corticotropin releasing hormone (CRH)	Stimulates secretion of ACTH
	Thyrotropin releasing hormone (TRH)	Stimulates secretion of TSH and prolactin
	Growth hormone releasing hormone (GHRH)	Stimulates secretion of GH
	Somatostatin (SS, also known as growth hormone release inhibiting hormone (GIH)	Inhibits secretion of GH and TSH (and possibly several other hormones)
	Gonadotropin releasing hormone (GnRH)	Stimulates secretion of LH and FSH
	Dopamine (DA, also known as prolactin release inhibiting hormone, PIH)*	Inhibits secretion of prolactin
	Posterior pituitary hormones	See posterior pituitary

Endocrine Gland	Hormone	Major Function of:
Anterior pituitary	Growth hormone (somatotropin, (GH)†	Growth via secretion of IGF-I, protein, carboydrate, and lipid metabolism
	Thyroid-stimulating hormone (TSH, thyrotropin)	Thyroid Gland
	Adrenocorticotropic hormone (ACTH, corticotropin)	Adrenal cortex
	Prolactin	Breast growth and milk synthesis; permissive for certain reproductive functions in the male
	Gonadotropic hormones Follicle-stimulating hormone (FSH) Luteinizing hormone (LH)	Gonads (gamete production and sex hormone secretion)
Posterior pituitary‡	Oxytocin	Milk let-down; uterine motility
	Vasopressin (antidiuretic hormone, ADH)	Water excretion by the kidneys, blood pressure
	Dopamine	Prolactin secretion
	Prolactin releasing factor	Prolactin secretion
Adrenal cortex	Cortisol	Organic metabolism, response to stresses, immune system
	Androgens	Sex drive in women

Endocrine Gland	Hormone	Major Function of:
Gonads.		
Female ovaries	Estrogen	Reproductive system, breasts; growth and development
	Progesterone	FSH secretion
	Inhibin	
	Relaxin	Relaxation of cervix and pubic ligaments
Kidneys	Renin (→angiotensin II)§	Aldosterone secretion, blood pressure
	Erythropoietin	Erythrocyte production
	1,25-dihydroxyvitamin D_3	Plasma calcium
Gastrointestinal tract	Somatostatin	Gastrointestinal tract, liver, pancreas, gallbladder
Liver (and other cells)	Insulin-like growth factors (IGF-I and II)	Growth
Thymus	Thymopoietin	T-lymphocyte function
Placenta	Chorionic gonadotropin (CG)	Secretion by corpus luteum
	Estrogens	See ovaries
	Progesterone	See ovaries
	Placental lactogen	Breast development; organic metabolism

*Dopamine is a catecholamine, all the other hypophysiotropic hormones are peptides

†The names and abbreviations in parentheses are synonyms

‡The posterior pituitary stores and secretes these hormones, they are made in the hypothalamus.

§Renin is an enzyme that initiates reactions in blood that generate angiotensin II

The nucleases suitable for use in the present invention include: ribonuclease, more specifically ribonuclease A, ribonuclease 1; ribonuclease A, oxidized; ribonuclease A, with scrambled disulfide bonds; ribonuclease S-peptide; ribonuclease S-protein; ribonuclease T_1; and ribonuclease T_2, ribonuclease B, ribonuclease C, ribonuclease H, ribonuclease S, ribonuclease T, ribonuclease U_1 and ribonuclease U_2. (The specific ribonucleases listed above are available from and

listed in Sigma Chemical Company's 1995 catalogue, pgs. 907-909, P.O. Box 14508, St. Louis, Missouri 63178). In addition, other nucleases, including those sometimes referred to as restriction enzymes can be used in the present invention. In addition, DNAse can also be used as a nuclease of the present invention, conjugated to desired hormones as mentioned elsewhere herein. Angiogenin can also be used in place of one of the designated nucleases and reference to nucleases herein is meant to include the use of angiogenin. Angiogenin is known to target tRNAs and is non-toxic outside of cells.

Preferably, nucleases are used that correspond to the genus and species of animals to be treated to minimize the immunogenicity of the hormone/nuclease conjugates and to confer maximum selectiveness of nucleases within such animals. It is possible, however, to use bacterial nucleases in mammals where immunogenic concerns are of lesser importance. Glycosolation of nucleases is preferred given the ability of carbohydrate groups to be used as potential conjugation sites for hormone linkages. It is within the scope of the present invention, however, to utilize deglycosolated nucleases conjugated to particular hormones.

In a most preferred embodiment, pancreatic ribonucleases are used which, like other ribonucleases, are toxic inside a cell but not outside of a cell. As such, in comparison with toxins described herein, nucleases, such as RNAse and DNAse, are better candidates for use in humans given the reduced concern over the administration of compounds deemed dangerous by the FDA and similar governmental agencies. RNAse does not normally get inside cells but is often secreted by cells. As such, RNAse from a particular genus and species is not immunogenic in that genus and species or in closely related genus and species. Given that the hormones conjugated to the nucleases

of the present invention are also endemic to animal systems, the hormones/nuclease conjugates of the present invention are far less immunogenic than the toxin conjugates elsewhere described herein.

Although the following discussion is directed to particular embodiments of the present invention, it should be understood that different hormones, (e.g., those listed on Table VI) and different nucleases can be conjugated and used in a manner similar to the particular hormone-nuclease conjugates described in detail below (e.g., doses, administration, targeting of desired cell types, etc.). In one preferred embodiment of the present invention, a GnRH analog is conjugated to RNAse, such conjugate linked together using one of the above-mentioned linking agents, or other linking agents deemed suitable by one of skill in the art based on the particular nuclease utilized. Linking agents may be capable of forming a carbon-nitrogen bond and can include the use of aldehydes, hydroxylamine, hydrazine, and derivatives thereof. Activated carboxyl groups can also be used to join nucleases to hormones. Preferably, the nucleic acid degrading agent (e.g., the RNAse and/or DNAse) is conjugated in a manner similar to that described above with respect to toxin conjugates. (See, e.g., Equation 1 above, substituting "N" (for nuclease), and more preferably RNAse and/or DNAse, for T). The GnRH/nuclease conjugate of the present invention can be administered to an animal in an effective manner according to individual dose size, number of doses, frequency of dose administration and mode of administration, as determined by particular protocols relating to the treatment of individual types of animals and based on the particular type of conditions sought to be treated. Determination of such a protocol can be accomplished by those skilled in the art without resorting to undue experimentation. An effective dose refers to a dose capable of treating a subject for a disorder as described herein, including a dose effective to achieve temporary and/or permanent sterility, a dose effective to incapacitate cells having GnRH receptors thereon, for the

purpose, for example, of inhibiting the secretion of particular compounds normally secreted by such cells, and for the treatment of abnormal cellular growth, such as cancers and tumors. As described above, an effective dose can be selected that destroys and/or incapacitates cells having GnRH receptors after the receptor is bound to the conjugate described herein. Effective doses can vary depending upon, for example, the therapeutic composition used, the medical disorder being treated and the size and type of the recipient animal. Effective doses to treat a subject include doses administered over time that are capable of regulating the activity, including growth, of cells involved in a medical disorder.

In one preferred embodiment, administration of GnRHa-RNAse A conjugate is performed either intravenously or intramuscularly. As the conjugate material enters the circulation, it will be carried to cells having GnRH receptors thereon, principally, if not solely, the anterior pituitary gland, where it will bind to receptors on the gonadotrophs. After binding to the receptor, the complex is internalized. Once inside the cell, the RNAse A will degrade cellular RNA, thus resulting in inhibition of protein synthesis. The lack of protein synthesis can result in cell death or the incapacitation of the cell to function in a normal capacity. Since gonadotrophs secrete hormones that stimulate the gonads, the incapacitation (e.g., death) of such gonadotrophs leads to gonadal atrophy and can result in permanent sterility. The only location of GnRH receptors in most species is on the gonadotroph, so there is not likely to be any side effects associated with such treatment. GnRH is the major hormone controlling reproduction in both males and females and specifically, in mammalian species. Therefore, the present invention is useful for sterilizing both sexes in a variety of species.

The use of GnRH/nuclease conjugates is preferred over the use of other toxin conjugates for several reasons. For example, because preferred nucleases used in conjunction with the present invention are produced by animals to be treated, immunogenic and allergic reactions are minimized and the prospect of treating animals with potentially harmful toxins is eliminated.

In particular, the use of RNAse A instead of the toxins described above has several potential advantages including: 1) RNAse A is smaller than many toxins, especially plant and bacterial toxins, so it is easier to specifically deliver to gonadotrophs; 2) RNAse A derived from or closely related to the species being treated can be used, thereby greatly reducing the changes of anaphylactic shock in the event the animal requires more than one treatment to achieve desired results (e.g., sterility); 3) much more is known about the structure of RNAse A than other toxins, thereby facilitating the conjugation of RNAse A to GnRHA; 4) RNAse A is far more stable than many toxins; and 5) RNAse is a glycoprotein and thus provides several different sites to conjugate without interfering with enzymatic activity. Because of such stability, GnRHA-RNAse A conjugates provide an increased effectiveness of such conjugates for desired uses, such as targeting cells having GnRH receptors.

One embodiment of the present invention, therefore, relates to GnRH/nuclease conjugates, and preferably a GnRH-RNAse conjugate. The term "RNAse" as used herein refers to any ribonucleic acid degrading compound, preferably RNAse found in the same animal that is to be treated with the hormone-RNAse conjugate of the present invention. To form a conjugate between an RNAse and GnRH, various linking agents can be used as described herein.

Similarly, in another embodiment, the present invention is directed to GnRH/DNAse conjugates. The term "DNAse" as used herein refers to any deoxyribonucleic acid degrading

compound. Conjugates between GnRH and DNAse can be formed using any of the above-mentioned linking agents.

Particularly preferred RNAse compounds affect RNA translation prior to any substantial amount of protein being produced by a cell. Particularly preferred DNAse compounds are capable of passing relatively easily through the nuclear membrane. It is within the scope of the present invention to utilize other agents to facilitate the transfer of a hormone/nuclease molecule across either a cell membrane or a nuclear membrane. Agents that facilitate access to cleavage sites on DNA and RNA molecules can also be used to bring about desired degradation of nucleic acid molecules.

The GnRH-RNAse conjugates of the present invention are preferably capable of crossing cell membranes of cells having GnRH receptors thereon. Such cells are principally those of the anterior pituitary gland, often referred to as gonadotrophs. Other cells having GnRH receptors, however, can be targeted using the compound of the present invention, such cells including cancer cells and undifferentiated cells that, at some stage during their life cycle, express GnRH receptors on their surface. While not bound by theory, it is believed that certain cancer cells express genes encoding for GnRH receptors and that such receptors are presented on the surface of such cells. As such, in appreciation of this fact, the present invention can be used to target cancer cells that present, at some stage during their life cycle, GnRH receptors on their surface.

As described in more detail above, the present invention can also be used to treat a variety of hormone related diseases, such as, but not limited to, diseases involving the target organs of hormones listed in Table VI, specifically including prostate cancer, fibroid tumors, breast cancer, endometriosis, Cushing's disease, acromegaly, giantism, melanomas and osteoporosis. It is within

the skill of the art to select particular hormone-nuclease conjugates to treat a variety of disease states and cell types associated therewith.

The present invention also includes a method for using GnRH-RNAse compounds as a non-surgical means of sterilizing both male and female animals, including humans. At the present time, there is no method available for permanent sterilization of animals, other than surgical removal of the gonads. In addition to the use of the present invention to sterilize domestic animals, which previously required spaying or neutering of such animals, the present invention can be used to treat a large variety of animal species including domestic livestock and wildlife species, such as deer, elk, feral horses, etc. The present invention thus affords a method for controlling the population of wildlife in areas where hunting is not permitted.

Previous methods for permanently sterilizing animals have been limited to surgical castration, vasectomy and/or tubal ligation. Although chemicals have been utilized to inhibit reproductive functions in animals, such chemicals often require repeated and/or continuous administration to ensure that such animals do not have the capacity to reproduce. Moreover, immunization of animals against various components of the reproductive system has been attempted, however, such methods required that antibody titers remained high so that the reproductive system was inhibited.

Conventional surgical techniques to sterilize animals are expensive and generally require that the subject be anesthetized, thus entailing the inherent dangers of such procedures. Moreover, surgical techniques are not feasible for non-domesticated animals. One of the major problems in the use of chemical sterilants is that such chemicals are often present in the tissues of the treated animal for extended periods of time. If treated animals happen to be the prey for other

species, especially endangered species, it is possible that the fertility of the endangered species that eats a treated animal may be hindered. Furthermore, chemical sterilants may not be suitable for use in animals that are used for human consumption, or in animals that are prey of endangered species. Immunization against a component of the reproductive system provides an effective means for inducing sterility in several species, however, without booster injections on periodic basis, it has been noted that fertility of such animals is very likely to return. Because yearly boosters are not feasible, for example, with wildlife species, prior methods of regulating fertility have not been deemed effective or feasible. In brief, the more often a treatment is required to inhibit fertility, the less practicable such method is and the less likely such method is to gain public acceptance. As such, the present invention satisfies a great need for a non-surgical method for sterilizing animals that can be easily administered and that results in permanent sterility of treated animals.

Another aspect of the present invention relates to a nucleic acid molecule that encodes a conjugate of the present invention comprising a hormone linked to a nuclease. The hormones most preferred are those that comprise a single continuous sequence of amino acids and having at least one of their ends (i.e, amino or carboxyl end) capable of being linked (e.g., covalently attached) to an amino acid sequence of a nuclease. These types of hormones are preferred since a single nucleic acid sequence can encode a particular hormone as well as a desired nuclease. Preferred hormones are therefore those that have a single chain, such hormones including: GnRH; prolactin, motilin, TRH, MSH, somatostatin, GHRH, CRH and ACTH. Although steroid hormones including estrogens, progestins, androgens, and corticosteroids, especially progesterone, testosterone, dihydrotestostrone, cortisol and estradiol, can be used in the present invention, they are not comprised of amino acid sequences and therefore are not encoded by nucleic acid molecules. Other

hormones, however, can be transcribed from nucleic acid molecules in more than one chain, for example, FSH, TSH, LH and HCG. In addition, antagonists that bind to the same receptor as any of the above-stated hormones can also be encoded on nucleic acid molecules. The present invention therefore includes the use of more than one nucleic acid molecule that encodes a particular hormone so that such hormone can be produced within a cell, or can be produced in separate cells and later combined to form a fully functional hormone which can then be linked to a nuclease to form a hormone-nuclease conjugate of the present invention.

According to the present invention, references to nucleic acids also refer to nucleic acid molecules. A nucleic acid molecule can be DNA, RNA, or hybrids or derivatives of either DNA or RNA. Nucleic acid molecules of the present invention can include regulatory regions that control expression of the nucleic acid molecule (e.g., transcription or translation control regions), full-length or partial coding regions, and combinations thereof. Specific nucleic acid sequences of particular hormones and nucleases can be obtained from GenBank and one of ordinary skill in the art can easily select the desired hormone-nucleic acid sequences available from GenBank, or another publicly available source, and covalently link (by base pair linkage) such sequences to nucleic acid sequences of desired nucleases, as otherwise set forth herein. Similarly, the amino acid sequences of any particular hormone and/or nuclease can be obtained from GenBank and such amino acid sequences can be conjugated together using the methods taught herein to form effective hormones/nuclease conjugates. Such conjugates can then be used to treat particular disease states involving cells having receptors capable of binding particular hormones.

Nucleic acid and amino acid sequences for particular hormones and for particular nucleases can be obtained from the GenBank directory available from the National Center for Biotechnology

Information, National Library of Medicine, National Institutes of Health, Building 38A, 8600 Rockville Pike, Bethesda, Maryland 20894. Given that the present inventors are the first to appreciate the usefulness of hormone-nuclease conjugates for the uses described herein, publicly available and enabling sequences for components of such conjugates, 3fragments.e.g., specific hormones for particular genus and species, as well as for particular nucleases, are not set forth herein because they are available from publicly accessible sources, such as GenBank, as described above. All nucleic acid and amino acid sequences for the hormones set forth in Table VI, as well as the nucleases described herein, are therefore incorporated herein by this reference.

A nucleic acid molecule of the present invention can be produced by: (1) isolating hormone and nuclease nucleic acid molecules from their natural milieu and joining them together; (2) using recombinant DNA technology (e.g., PCR amplification, cloning); or (3) using chemical synthesis methods. A nucleic acid of the present invention can include functional equivalents of natural nucleic acid molecules encoding hormone-nuclease conjugates including, but not limited to, natural allelic variants and modified nucleic acid molecules in which nucleotides have been inserted, deleted, substituted, and/or inverted in such a manner that such modifications do not substantially interfere with the nucleic acid molecule's ability to encode a hormone-nuclease conjugate of the present invention. Preferred functional equivalents include sequences capable of hybridizing under stringent conditions, to at least a portion of a full length hormone/nuclease molecule encoding nucleic acid molecule (according to conditions described in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Labs Press, 1989, which is incorporated herein by reference in its entirety). Preferably the length of a particular nucleic acid sequence is sufficient to encode at least 15 amino acids. As guidance in determining what particular modifications can be made to any

particular nucleic acid molecule, one of skill in the art should consider several factors that, without the need for undue experimentation, permit a skilled artisan to appreciate workable embodiments of the present invention. For example, such factors include modifications to nucleic acid molecules done in a manner so as to maintain particular functional regions of the encoded proteins including, a working hormone cell binding domain, a functional nuclease domain, and in particular embodiments, a linking agent that does not substantially interfere with desired binding interactions between a particular hormone and a target cell and/or that does not compromise the enzymatic activity of a linked nuclease. Functional tests for these various characteristics (e.g., binding and/or nuclease activity studies) allows one of skill in the art to determine what modifications to nucleic acid sequences would be appropriate and which would not.

One embodiment of the present invention includes a nucleic acid molecule encoding a hormone-nuclease molecule having at least three components: (1) a hormone segment; (2) a nuclease component; and (3) a linking agent that encodes for a protein capable of conjugating a hormone segment to a nuclease component. Suitable and preferred hormone segments, nucleases, and linking agents for use in the present invention are heretofore disclosed. A nucleic acid molecule of the present invention comprises at least one nucleic acid sequence encoding a hormone, covalently attached (by base pair linkage) to at least one nucleic acid sequence encoding a nuclease component. The nucleic acid sequences are attached in such a manner that the sequences are transcribed in-frame, thereby producing a functional hormone-nuclease molecule capable of targeting specific cells having receptors for such hormones.

Preferred nucleic acid molecules encoding hormone-nuclease conjugates include: those nucleic acid molecules encoding hormones known to have at least one of their amino and/or carboxyl

ends available for attachment to a nuclease amino acid sequence, wherein such attachment does not significantly affect the capability of the particular hormone to bind to cells having receptors for such a hormone. Hormones that do not undergo post-translational modification are preferred, thus enabling the transcription of one length of a nucleic acid molecule encoding a desired hormone and a desired nuclease. Most hormones have the above characteristics, although TSH, HCG, LH, FSH and GnRH are exceptions. These later hormones are thus conjugated to desired nucleases after post-translational modification of such hormones.

To facilitate production of hormone-nuclease conjugates, nucleic acid molecules encoding desired hormone-nuclease conjugates may also comprise a nucleic acid sequence that encodes for a signal or leader segment that is capable of promoting secretion of conjugates from the cell that produces them. Nucleic acid sequences encoding the leader or signal segments are covalently associated (by base pair linkage) to the 5' end of a nucleic acid molecule. The leader or signal segments can be segments which naturally are associated with a hormone or a particular nuclease.

Another embodiment of the present invention is a fusion protein that includes a hormone-nuclease molecule containing-domain attached to a fusion segment. Inclusion of a fusion segment as part of a hormone-nuclease molecule of the present invention can enhance the molecule's stability during production, storage and/or use. Furthermore, a fusion segment can function as a tool to simplify purification of a hormone-nuclease molecule, such as to enable purification of the resultant fusion protein using affinity chromatography. A suitable fusion segment can be a domain of any size that has the desired function (e.g., increased stability and/or purification tool). It is within the scope of the present invention to use one or more fusion segments. Fusion segments can be joined to amino and/or carboxyl termini of the hormone-nuclease molecule. Linkages between fusion

segments and a hormone-nuclease molecule can be made to be susceptible to cleavage to enable straight-forward recovery of the hormone-nuclease molecules. Fusion proteins are preferably produced by culturing a recombinant cell transformed with a fusion nucleic acid sequence that encodes the fusion segment attached to either the carboxyl and/or amino terminal end of a hormone-nuclease conjugate.

A separate embodiment of the present invention comprises the use of particular hormones conjugated to pieces or fragments of nuclease molecules. Nuclease fragments conjugated to such hormones will be targeted to specific cells having receptors for the corresponding hormone, thus permitting the nuclease fragments to pass through the cell membrane into the cell. Once in the cell, the nuclease fragments can reassemble to form active nuclease molecules, and thus can degrade nucleic acid molecules within the cell, resulting in the incapacitation and destruction of such targeted cells. One of ordinary skill in the art will possess requisite knowledge required to determine particular enzymes that can be used to cut up nucleases, such as RNAse, in order to form the above-referenced fragments (e.g., S-peptides, etc.). The above-referenced fragments can be conjugated to hormones having particular cell binding domains in a fashion described elsewhere in the present application. Modified catalytic portions of nucleases capable of forming catalytic competent complexes can thus be conjugated to either full length hormones or the cell binding domains of particular hormones so that, once targeted to particular cells, such modified catalytic portions can reassemble to function as effective nuclease agents.

While not bound by theory, it is believed that conjugating a hormone to a nuclease may actually make the hormone conjugate more potent due to the increase in length of the molecule. Such increased length is believed to protect the molecule from being secreted from the body and

thus, the clearance rate of the hormone-nuclease conjugate should be reduced. Moreover, because the hormone-nuclease conjugates will have an increased half-life, doses of such conjugates can be drammatically reduced as compared to the doses of hormones conventionally delivered to treating individuals. The nuclease domain conjugated to the hormone is also believed to enhance the stability of the hormone, and thus the entire conjugate itself is a more stable molecule.

The present invention also includes a recombinant molecule comprising a nucleic acid sequence encoding a hormone-nuclease molecule operatively linked to a vector capable of being expressed in a host cell. As used herein, "operatively linked" refers to insertion of a nucleic acid sequence into an expression vector in such a manner that the sequence is capable of being expressed when transformed into a host cell. As used herein, an "expression vector" is an RNA or DNA vector capable of transforming a host cell and effecting expression of an appropriate nucleic acid sequence, preferably replicating within the host cell. Construction of desired expression vectors can be performed by methods known to those skilled in the art and expression can be in eukaryotic or prokaryotic systems. Procaryotic systems typically used are bacterial strains including, but not limited to various strains of *E. coli*, various strains of *bacilli* or various species of Pseudomonas. In prokaryotic systems, plasmids are used that contain replication sites and control sequences derived from a species compatible with a host cell. Control sequences can include, but are not limited to promoters, operators, enhancers, ribosome binding sites, and Shine-Dalgarno sequences. Expression systems useful in eukaryotic host cells comprise promoters derived from appropriate eukaryotic genes. Useful mammalian promoters include early and late promoters from SV40 or other viral promoters such as those derived from baculovirus, polyoma virus, adenovirus, bovine papilloma virus or avian sarcoma virus. Expression vectors of the present invention include any vectors that

function (i.e., direct gene expression) in recombinant cells of the present invention including bacterial, yeast, other fungal, insect, and mammalian cells. Particularly preferred expression vectors of the present invention include dual promoter baculovirus transfer vectors, and vectors containing class II promoters, β-actin promoters, globin promoters, or epithelial cell specific promoters.

An expression system can be constructed from any of the foregoing control elements operatively linked to the nucleic acid sequences of the present invention using methods known to those of skill in the art. (see, for example, Sambrook et al., *ibid.*)

Host cells of the present invention can be: cells naturally capable of producing particular hormones; or cells that are capable of producing hormone-nucleases when transfected with a nucleic acid molecule encoding a particular hormone-nuclease. Host cells of the present invention include, but are not limited to bacterial, yeast, fungal, insect and mammalian cells.

In one aspect of the present invention, recombinant cells can be used to produce at least one hormone-nuclease molecule by culturing such cells under conditions effective to produce such molecules, and recovering the molecules. Effective conditions to produce a recombinant molecule include, but are not limited to appropriate culture media, bioreactor, temperature, pH and oxygen conditions. Depending on the expression vector used for production, resultant molecules can either remain within the recombinant cell, be retained on the outer surface of the recombinant cell, or be secreted into the culture medium.

It has also been found effective to use protein inhibitors of nucleases, in particular inhibitors of ribonuclease, to protect cells used to produce such nucleases. For example, genes for inhibitors of ribonuclease can be incorporated into host cells and expression of such genes results in the

production of inhibitors to protect cells from "leaks" of nucleases that would otherwise be toxic to cells used in production systems.

As used herein, the term "recovering the conjugate" refers to collecting the fermentation medium containing the conjugate and/or recombinant cells. Recovery need not imply additional steps of separation or purification. Hormone-nuclease molecules of the present invention can be purified using a variety of standard protein purification techniques such as, but not limited to affinity chromatography, ion exchange chromatography, filtration, centrifugation, electrophoresis, hydrophobic interaction chromatography, gel filtration chromatography, chromatofocusing and differential solubilization. Isolated hormone-nuclease molecules are preferably retrieved in "substantially pure" form. As used herein, "substantially pure" refers to a purity that allows for the effective use of the molecule as a pharmaceutical composition or experimental reagent.

Soluble hormone-nuclease molecules of the present invention can be purified using, for example, immunoaffinity chromatography. Hormone-nuclease molecules anchored in a lipid-containing substrate can be recovered by, for example, density gradient centrifugation techniques.

One aspect of the present invention relates to the use of hormone-nuclease conjugates as formulations for therapeutic use, and can also be used to produce a pharmaceutical reagent. Such pharmaceutical reagents are useful for administration to patients suffering from diseases that are treatable by destroying or otherwise compromising the activity of cells that bind a particular hormone. The hormone-nuclease conjugates can also be used to sterilize individuals by destroying select cells targeted by particular hormones. For example, Sertoli cells that produce sperm bind FSH. FSH-nuclease conjugates can thus be used to destroy Sertoli cells that bind FSH-nuclease conjugates. Partial destruction of such cells may be sufficient to temporarily sterilize the male since

insufficient amounts of sperm may be produced. Total destruction of such cells can be used to permanently sterilize males without detracting from otherwise normal sexual function.

Pharmaceutical reagents of the present invention can be administered to any animal, preferably to mammals, and even more preferably humans. Acceptable protocols to administer pharmaceutical formulations in an effective manner include individual dose size, number of doses, frequency of dose administration, and mode of administration. Modes of delivery can include any method compatible with prophylactic or treatment of a disease. Modes of delivery include, but are not limited to, parenteral, oral, intravenous, topical or local administration such as by aerosol or transdermally. A pharmaceutical reagent of the present invention is useful for the treatment of any hormone-related disease that is susceptible of treatment by destruction (e.g., killing of cells) that have receptors for specific hormones.

Yet another aspect of the present invention involves the use of antibodies bound to DNAse, such antibodies capable of targeting specific cells having ligands on the surfaces thereof capable of binding to such antibodies. Methods for linking or otherwise conjugating antibodies to DNAse will be appreciated by those of skill in the art. The binding of the antibodies/DNAse molecules of the present invention by a cell will result in the incorporation of the antibody/DNAse conjugate into the cell. The DNAse thus delivered can pass through the nuclear membrane and degrade DNA, thereby resulting in the destruction or incapacitation of the antibody targeted cell.--

IN THE CLAIMS:

Please cancel Claims 1-8 without prejudice to or disclaimer of the subject matter contained therein and add the following new claims:

9. (Added) A method for using at least one hormone/toxin conjugate to sterilize an animal, said conjugate comprising a peptide hormone capable of binding to a GnRH receptor, conjugated by a linking agent to a toxin group selected from the group consisting of a chemical toxin, a single chain toxin, and a modified toxin having an intrinsic toxic group lacking a functional binding domain, said conjugate capable of selectively binding to a gonadotroph and of substantially precluding said gonadotroph from secreting gonadotropins, said method comprising administering an effective amount of said conjugate to said animal to substantially preclude secretion of gonadotropins by said animal's gonadotrophs, wherein said conjugate is capable of crossing the cell membrane of a gonadotroph wherein said peptide hormone has the general formula

pyroGlu-His-Trp-Ser-Tyr-X-Leu-Arg-Pro,

wherein X is an amino acid selected from the group consisting of lysine, D-lysine, ornithine, D-ornithine, glutamic acid, D-glutamic acid, aspartic acid, D-aspartic acid, cysteine, D-cysteine,

tyrosine and D-tyrosine.

10. (Added) The method of Claim 9, wherein said method is effective to temporarily sterilize said animal.

11. (Added) The method of Claim 9, wherein said peptide hormone is GnRH or an analog thereof wherein said peptide hormone has the general formula

pyroGlu-His-Trp-Ser-Tyr-X-Leu-Arg-Pro,

wherein X is an amino acid selected from the group consisting of lysine, D-lysine, ornithine, D-ornithine, glutamic acid, D-glutamic acid, aspartic acid, D-aspartic acid, cysteine, D-cysteine, tyrosine and D-tyrosine.

12. (Added) The method of Claim 9, wherein said peptide hormone has GnRH-ethylamide.

13. (Added) The method of Claim 9, wherein said toxin group comprises a recombinantly produced protein that inhibits biosynthesis.

14. (Added) The method of Claim 9, wherein said modified toxin is selected from the group consisting of modified ricin toxins, modified modeccin toxins, modified abrin toxins, modified diphtheria toxins, modified Pseudomonas exotoxins and modified shiga toxins.

15. (Added) The method of Claim 9, wherein said single chain toxin is selected from the group consisting of pokeweed antiviral protein, α-amanitin, gelonin ribosome inhibiting protein ("RIP"), barley RIP, wheat RIP, corn RIP, rye RIP, flax RIP, and modified forms thereof.

16. (Added) The method of Claim 9, wherein said chemical toxin is selected from the group consisting of melphalan, methotrexate, nitrogen mustard, doxorubicin and daunomycin.

17. (Added) A method of Claim 9, wherein said toxin group is selected from the group consisting of modified diphtheria toxins and modified Pseudomonas exotoxins, wherein said toxin group comprises a toxic domain and a translocation domain but lacks a functional toxin cell binding domain.

18. (Added) The method of Claim 9, wherein said linking agent is selected from the group consisting of 2-iminothiolane, N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), 4-succinimidyloxycarbonyl-α-(2-pyridyldithio)-toluene (SMPT), m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), N-succinimidyl(4-iodoacetyl)aminobenzoate (SIAB), succinimidyl 4-(p-maleimidophenyl)butyrate (SMPB), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), bis-diazobenzidine and glutaraldehyde.

19. (Added) The method of Claim 9, further comprising challenging said animal with GnRH at least about four weeks after said step of administering said conjugate to said animal, said challenging not inducing substantial secretion of luteinizing hormone by said animal.

20. (Added) The method of Claim 19, wherein said method of administering said conjugate is repeated at least once over a time period such that administration of said conjugate does not elicit a substantial production of antibodies against said conjugate.

21. (Added) A method for using at least one hormone/toxin conjugate to functionally inactivate cells whose membranes contain receptors for GnRH, said conjugate comprising a peptide hormone capable of binding to a GnRH receptor conjugated by a linking agent to a toxin group, said method comprising administering an effective amount of said conjugate to an animal to chemically attack said cells, and wherein said peptide hormone has the general formula

pyroGlu-His-Trp-Ser-Tyr-X-Leu-Arg-Pro,

wherein X is an amino acid selected from the group consisting of lysine, D-lysine, ornithine, D-ornithine, glutamic acid, D-glutamic acid, aspartic acid, D-aspartic acid, cysteine, D-cysteine, tyrosine and D-tyrosine.

22. (Added) A method for functionally inactivating gonadotrophs in the pituitary gland of an animal, comprising administering to said animal an effective amount of a hormone/toxin conjugate comprising a peptide hormone conjugated to a toxin group, wherein said conjugate is capable of selectively binding with receptors on said gonadotrophs to render said gonadotrophs essentially incapable of secreting gonadotropins, wherein said animal is not weakened or killed by said method and wherein said peptide hormone has the general formula

pyroGlu-His-Trp-Ser-Tyr-X-Leu-Arg-Pro,

wherein X is an amino acid selected from the group consisting of lysine, D-lysine, ornithine, D-ornithine, glutamic acid, D-glutamic acid, aspartic acid, D-aspartic acid, cysteine, D-cysteine, tyrosine and D-tyrosine.

REMARKS

Applicants' counsel requests the courtesy of a telephone interview to address any questions or concerns the Examiner may have. Applicants' counsel can be reached directly at (303) 863-2977.

Respectfully submitted,

SHERIDAN ROSS P.C.

By: _____

Joseph E. Kovarik
Registration No. 33,005
1560 Broadway, Suite 1200
Denver, Colorado 80202-5141
(303) 863-9700

Date: 1/21/02

J:\2730\-97-con\preliminary amendment wpd